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13. ABSTRACT *(Maximum 200*

During the third year of our studies of I3C and its products, we found, **A.** At pharmacologic concentrations, DIM induces apoptosis in breast tumor cells that is accompanied by a loss of cytosolic Bcl-2 protein. **B.** CTr-1 is a strong agonist of estrogen receptor function (1). **C.** Ascorbigen is an inhibitor of CYP1A1 and is converted in cell culture to a strong Ah agonist (2). **D.** NI3C is a more efficient inducer of CYP1A1 in cultured cells but less active when administered to rodents (3). **E.** In the NCI tumor cell culture screen, pronounced antiproliferative effects required concentrations of DIM of greater than 10 μM and of LTr-1 of greater than 1 μM. **F.** Gene expression microarray analysis of mRNA from DIM-treated breast tumor cells indicates that all five of the most strongly down-regulated genes for which function is established are members of gene families important in processes involved in tumor angiogenesis. Our results show that, contrary to our expectations, the primary effect of the major in vivo products of I3C, i.e. DIM and CTr-1, was to activate ER function and to promote proliferation of estrogen-dependent cells in culture and implicate DIM in the control of tumor angiogenesis.

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Introduction:

Indole-3-carbinol (I3C) is a naturally occurring component of dietary vegetables and a promising cancer preventive agent, most notably against breast cancer. I3C markedly reduces the incidence of spontaneous and carcinogen-induced mammary tumors in rodents and exhibits potent growth inhibitory activity in human breast cancer cells. Although I3C has reached the stage of phase I clinical trials, little is known about the mechanism of its growth inhibitory effects in cancer cells. The purpose of this work is to establish the mechanism of action and to exploit the cancer preventive properties of I3C and related compounds.

I3C is active in several key anticancer-related bioassays. Rodents exposed to high doses of I3C via oral intubation or diet exhibited increases in the activities of a variety of cytochrome P-450-dependent activities including hepatic ethoxyresorufin O-deethylase (EROD) and related activities (4,5). I3C reduced BP-induced neoplasia of the forestomach (6) and total covalent binding of BP and N-nitrosodimethylamine to hepatic DNA in mice (7,8,9). In trout, I3C reduced AFB1-induced hepatocarcinogenesis when administered prior to and during carcinogen treatment (10). In a recent screen of 90 potential chemopreventive agents in a series of 6 short-term bioassays relevant to carcinogen-induced DNA damage, tumor initiation and promotion, and oxidative stress, I3C was found to be one of only 8 compounds that tested positive in all assays. The authors of this study opined that I3C was highly promising for development as a cancer chemopreventive agent (11).

Some of the most well established biological effects of I3C appear to be related to its antiestrogenic effects. In a long term feeding experiment, in which female mice consumed synthetic diets containing I3C at 0, 500 or 2000 p.p.m., spontaneous mammary tumor incidence and multiplicity were significantly lower (ca. 50% reduction) at both doses of I3C than for untreated control animals, and tumor latency was prolonged in the high dose group (12). Oral administration of I3C to humans at doses of around 500 mg daily for one week produced an increase in estradiol 2-hydroxylation of approximately 50% in both men and women (13). I3C also increased the levels of estradiol hydroxylation activity in female rats (14).

The effects of I3C on DMBA-induced mammary tumors in rodents were reported in two studies. Wattenberg reported that I3C administered in the diet or by oral intubation prior to treatment with carcinogen reduced tumor incidence by 70-80% (6). In a recent study by Lubet, I3C administered prior to and during DMBA treatment reduced mammary tumor incidence by as much as 95% in rats (15). In a post-initiation protocol, I3C administration following treatment with NMU reduced tumor incidence by 65% (15). Consistent with these results, supplementation of a purified diet with cabbage or broccoli, both of which vegetables are good sources of I3C, also resulted in decreased mammary tumor formation in DMBA-treated rats (16).

Wattenberg also examined the cancer-protective effects of the predominant I3C conversion product, 3,3'-diindoylmethane (DIM). The results indicated that DIM was also highly effective in reducing DMBA-induced mammary tumors (6). Safe et al., using a protocol in which DIM treatment was initiated after DMBA-induced tumors had begun to develop, showed that treatment of rats every other day with the indole in a dose of only <u>5 mg/kg</u> dramatically inhibited further growth of the tumors (17). This dose of DIM did not induce CYP1A1 activity in the rats, which indicates that the Ah receptor pathways were not activated. On the assumption that DIM is excreted at a reasonable rate, this dose would be expected to produce blood concentrations of DIM of no more than 1-2 μM.

The antiestrogenic growth suppressive effects of I3C are also established for breast tumor cells in culture. Estradiol-induced proliferation of high density human MCF-7 cells was totally blocked by 50 μM I3C in the growth medium. I3C did not affect the proliferation of the estrogen-independent breast tumor line MDA-MB-231 in this experiment (18).

Because of these well documented cancer protective effects of I3C, along with its effects on estrogen metabolism, its low toxicity, and its wide availability, I3C is currently undergoing at least two different phase I clinical trials as a cancer chemotherapeutic and preventive agent (15).

Our working hypothesis was that I3C is converted in gastric acid to products that stimulate changes in the expression and activity of a network of early-response regulatory molecules that control a subsequent cascade of events leading to the arrest of human mammary tumor cell growth. We suggest further that one result of activation of this cascade is the blockade of estrogen-induced signal transduction pathways central to cell growth and proliferation.

Body:

1. Results and Discussion.

Progress was made under each of the stated objectives.

I.Identify I3C products that are responsible for I3C's growth inhibitory effects in breast tumor cells. Our continued studies in this area concentrated on the effects of DIM in breast tumor cells and in a broad array of human tumor cell lines at part of an NCI screen.

a. DIM induces apoptosis in both MCF-7 and MDA-MB-231 cells. We observed that incubation of mammary tumor cell lines MCF-7 and MDA-MB-231 in complete medium with DIM at concentrations of greater than 10 μM produced cell death. We determined that DIM induced apoptosis in both cell lines. Apoptosis was characterized by cellular and nuclear shrinkage, chromatin condensation, DNA fragmentation, proteolytic cleavage of certain proteins and phosphatidylserine externalization. Annexin V assay indicated that after only 10 min of treatment with 100 μM DIM, the characteristic halo image of phosphatidylserine (PS) externalization could be observed by fluorescent microscopy, indicating early stage of apoptosis. By 3 hours of treatment with 50 μM DIM, we observed both early stage and late stage of apoptosis cells. Chromatin condensation, measured with propidium iodine and Hoechst 33342 staining, was apparent by 24 hours of incubation with 50 μM DIM. DNA fragmentation, as measured by the ApopAlert DNA Fragmentation assay was observed by fluorescence microscopy in cells treated with 50 μM DIM. Quantification with flow cytometry indicated that both DIM (100 μM) and TAM (25 μM) induced DNA fragmentation in about 60% of cells. Our continuing mechanistic studies indicate that DIM induced a rapid loss of Bcl-2 protein and an increased expression of TGF-ß. Taken together, these results indicate that pharmacological concentrations of DIM can induce apoptosis by a mechanism similar to tamoxifen and vitamin E succinate in terms of TGF-ß induction and to taxol in terms of drop in levels of Bcl-2.

b. NCI screen of antiproliferative effects of DIM in cancer cell lines. DIM, LTr-1 and several other synthetic or natural products of I3C were submitted to the NCI for screening in 59 tumor cell lines (Figure 1). For these assays, cells are grown in serum-rich medium appropriate for each line. As indicated in Appendix B, although LTr-1 exhibited some selectivity for certain cell lines, especially with the melanoma group, pronounced antiproliferative activity in nearly all the lines required concentrations of greater than 1 μM. LTr-1 was clearly toxic to most lines at concentrations above 10 μM. With the exception of one of the non-small cell lung cancer lines, in which DIM produced a nearly 50% inhibition of proliferation at a concentration of only 0.1 μM, DIM was about an order of magnitude less active than LTr-1 in the assays. On the basis of these results, neither of these substances was considered suitable for further testing by the NCI.

II. Characterize effects of I3C products on estrogen receptor- and Ah receptor-mediated cellular responses.

a. The major cyclic trimeric product of I3C is a strong agonist of the estrogen receptor signaling pathway. We observed that 5,6,1,1,12,17,18-hexahydrocyclona[1,2-b:4,5-b':7,8-b"]triindole (CTr) stimulated the proliferation of estrogen-responsive MCF-7 cells in estradiol (E2-) depleted medium to a level similar to that produced by estradiol (E2), but did not affect the growth of the estrogen-independent cell line MDA-MD-231 in complete medium (Figure 2). CTr displaced E2 in competitive binding studies and activated ER binding to an estrogen-responsive DNA element in gel mobility shift assays with an EC50 of about 0.1 μM (Figure 3,4). CTr activated transcription of an E2-responsive endogenous gene and exogenous reporter genes in transfected MCF-7 cells, also with high potency (Figure 5). CTr (1 μM) failed to activate AhR-mediated pathways, consistent with the low binding affinity of CTr for the AhR reported previously (Figure 6). Comparisons of the conformational characteristics of CTr with other ER ligands indicated a remarkable similarity with tamoxifen (Figure 7). These studies have uncovered a new class of strong ER agonists that might be the source of the cancer-promoting activity of high oral doses of I3C seen in some experiments.

b. Modulation of CYP1A1 activity by ascorbigen in murine hepatoma cells. Ascorbigen (ASG) is the predominant indole formed during the degradation of glucobrassicin in plants. The major focus of this study was to examine the effects of ASG on CYP induction in a murine hepatoma-derived cell line (Hepa-1c1c7). ASG induced ethoxyresorufin O-deethylase (EROD) activity, a marker for CYP1A1, in a concentration dependent manner with maximum induction at 700 μM (Figure 8). Maximum ASG induction after 24 h treatment was 7% of maximal CYP1A1 activity induced indolo[3,2-b]carbazole (ICZ) (1 μM). Surprisingly, the CYP1A1 activity continuously increased up to 72 h when ASG had the same induction potency as 1 μM ICS after only 24 h. (Figure 9). CYP1A1 protein level, measured by Western blot analysis, was maximally induced after 24-h incubation with ASG. ASG was a potent inhibitor of CYP1A1 enzyme activity above 50 μM. ASG increased the chloramphenicol acetyl transferase (CAT) activity of a CAT reporter construct containing a dioxin responsive element (DRE) in transfected Hepa-1c1c7 cells, indicating activation of the Ah receptor.

c. N-Methoxyindole-3-carbinol is a more efficient inducer of CYP1A1 in cultured cells than I3C. The purpose of these experiments was to determine the effects of N-methoxyindole-3-carbinol (NI3C) on CYP1A1 induction in cultured cells and to compare the CYP-inducing potential of NI3C and I3C administered to rats. NI3C induced EROD activity in Hepa-1c1c7 cells in a concentration-dependent manner with 10-fold higher efficiency than I3C. As NI3C induced binding of the AhR to the dioxin-responsive element and induced expression of both endogenous CYP1A1 mRNA and an AhR-responsive chloramphenicol acetyl transferase construct, we concluded that NI3C could activate the AhR. Besides the induction of CYP1A1, we observed an inhibition of EROD activity, with an IC50 of 6 μM. Oral administration of 570 μm of NI3C/kg b.w. to male Wistar rats increased the hepatic CYP1A1 and 1A2 protein levels, as well as the EROD and 7-methoxyresorufin O-demethylase activities at 8 and 24 h after administration, but the responses were less pronounced than after administration of 570 μmol I3C/kg b.w.. Furthermore, NI3C did not induce hepatic 7-pentoxyresorufin O-depentylase activity whereas I3C did. Ascorbigen only weakly induced hepatic CYP1A1 and 1A2 but had no effect on CYP2B1/2. Compared to I3C, NI3C is a more efficient inducer of CYP1A1 in cultured cells but less active when administered to rodents.

III. Identify genes involved in the I3C-mediated inhibition of growth of breast tumor cells.

Gene expression microarray analysis of DIM-treated breast tumor cells. Gene expression microarray analysis of mRNA from MCF-7 cells treated with 50 μM DIM compared to vehicle-treated controls indicated that after 24 hr., of the 9800 sequences examined, the expression of 31 genes was induced by at least 2.0 fold and expression of 39 genes was decreased by at least 2.0 fold. The up-regulated group includes two weakly induced AhR-responsive genes, consistent with the previous findings by us and others that DIM is a weak activator of Ah receptor functions (19). Of possible significance in this group of induced genes is the activation of several interferon-responsive genes, including a 3.3-fold increase in expression of 2'-5'oligoadenylate synthetase gene, the product of which is essential for the antiviral activity of the interferons (20). This result raises the intriguing possibility of a role of DIM in the regulation of pathways responsible for the antiproliferative and antiangiogenic activities of cytokines (21).

For the down-regulated group of genes, a much greater association with a significant cancer-protective process is apparent. For this group, all eight of the most strongly repressed genes are important cell growth-related transcription factors and/or are associated with angiogenic processes.

a. Down-regulated transcription factors. The most strongly down-regulated gene, **NAK1/TR3** orphan receptor, is a member of the steroid/thyroid hormone receptor superfamily of transcription factors. Although the function of this gene product is poorly understood. NAK1/TR3 is the human homologue of the proteins encoded by the rat NGFI-B and mouse nur77 genes. These genes are induced rapidly by androgens and growth factors and may have functions related to cell proliferation, differentiation, and apoptosis. The nur77 protein product has many important functions related to cell proliferation including involvement in the signal transduction pathways mediated by retinoic acid, growth factors, and phorbol esters (22).

The immediate-early gene, **Egr-1**, produces a zinc finger protein involved in many cellular processes including the regulation of nur77 and of platelet-derived growth factor, the latter of established importance in the maintenance of normal vascular homeostasis (23). Hyaluronan-mediated activation of Egr-1 expression promotes angiogenesis in bovine aortic endothelial cells (24). Egr-1 is reported to be co-regulated with c-fos in several tumor lines, as we observed in DIM-treated MCF-7 cells. Although the function of **Egr-2** is poorly understood, one process in which this close relative of Egr-1 is implicated is the regulation of the expression of the pro-apoptotic Fas ligand (25). The nuclear oncogene, **c-fos**, has many important functions in the cell including activation of c-fos-induced growth factor/vascular endothelial growth factor D (Figf/Vegf-D) which mediates angiogenesis in several assay systems (26). **FosB/G0S3**, another component of the AP-1 family of transcription factors, is up regulated during apoptosis in breast tumor-derived cells (27). Following is a listing of the 39 most strongly induced genes.

Induced genes (fold induction)

5.3# Homo sapiens Chromosome 16 BAC clone CIT987SK-A-575C2 {Incyte PD: 3721987}
3.5 3-phosphoglycerate dehydrogenase {Incyte PD: 998612}

3.4 anterior gradient 2 (Xenopus laevis, secreted cement gland) homologue {Incyte PD:1998428}
3.3 cytochrome P450, subfamily I (dioxin-inducible), polypeptide 1 (glaucoma 3, primary infantile) {Incyte PD: 719318}
3.3 2'-5'oligoadenylate synthetase 2 {Incyte PD: 3214930}
3.2 cytochrome P450, subfamily I (aromatic compound-inducible), polypeptide 1 {Incyte PD: 2933775}
3.0 solute carrier family 7 (cationic amino acid transporter, y+system), member 5 {Incyte PD: 1911012}
2.9 Homo sapiens mRNA expressed in osteoblast, complete cds{Incyte PD: 2537863}
2.8 interferon-induced, hepatitis C-associated microtubular aggregate protein (44kD) {Incyte PD: 1922658}
2.8 heat shock 70kD protein 5 (glucose-regulated protein, 78kD) {Incyte PD: 2884613}
2.7 prostate differentiation factor {Incyte PD: 2042056}
2.7 Human mRNA for 56-KDa protein induced by interferon{Incyte PD: 1215596}
2.5 hyaluronan synthase 2 {Incyte PD: 3602403}
2.5 phosphoenolpyruvate carboxykinase 2 (mitochondrial) {Incyte PD: 1631511}
2.4 insulin induced gene 1 {Incyte PD: 1712592}
2.3 calreticulin {Incyte PD: 2970280}
2.3 antigen identified by monoclonal antibodies 4F2, TRA1.10, TROP4, and T43 {Incyte PD: 2852561}
2.3 tyrosyl-tRNA synthetase {Incyte PD: 1559756}
2.2 calmegin {Incyte PD: 2498216}
2.2 malic enzyme 1, soluble {Incyte PD: 2622181}
2.1 argininosuccinate synthetase {Incyte PD: 1981145}
2.1 H.sapiens mRNA for 23 kD highly basic protein {Incyte PD: 2824181}
2.1 seryl-tRNA synthetase {Incyte PD: 821141}
2.1 methionine-tRNA synthetase {Incyte PD: 1671740}
2.0 ESTs {Incyte PD: 1368834}
2.0 ribosomal protein L17 {Incyte PD: 747335}
2.0 glucose regulated protein, 58kD {Incyte PD: 1932086}
2.0 methylene tetrahydrofolate dehydrogenase (NAD+ dependent), methenyltetrahydrofolate cyclohydrolase {Incyte PD: 2455118}
2.0 Homo sapiens exportin t mRNA, complete cds {Incyte PD: 1806542}
2.0 ESTs {Incyte PD: 2112348}
2.0 ferritin, light polypeptide {Incyte PD: 2868138}
2.0 alanyl-tRNA synthetase {Incyte PD: 1985161}
1.9 ribosomal protein L4 {Incyte PD: 4082816}
1.9 H.sapiens mRNA similar to Xenopus laevis mRNA for KDELreceptor {Incyte PD: 1856520}
1.8 interferon, alpha-inducible protein 27 {Incyte PD: 2900277}
1.8 transducin (beta)-like 1 {Incyte PD: 1685482}
1.8 interferon-induced protein 17 {Incyte PD: 2902903}

b. Down-regulated cell adhesion and extracellular matrix proteins. The **S1-5** gene product is a member of the fibulin family of extracellular matrix proteins, one member of which, fibulin-1, decreases migration of ovarian tumor cells (28). Human connective tissue growth factor (**CTGF**) and insulin-like growth factor binding protein 10/**Cyr61** belong to a family of secreted proteins involved in cell adhesion, migration and mitogenesis in both fibroblasts and endothelial cells. Cyr61 was identified as an angiogenic inducer that can promote tumor growth and vascularization (29). Following is a listing of the 33 most strongly down-regulated genes.

Inhibited genes (fold inhibited)

12.2 Hormone receptor (growth factor-inducible nuclear protein N10) {Incyte PD:1958560}
5.3 Early growth response protein 1{Incyte PD:1705208}
5.1 Connective tissue growth factor{Incyte PD:1674454}
4.1 Homo sapiens Cyr61 mRNA, complete cds {Incyte PD:1514989}
4.1 EST{Incyte PD: 674714}
3.0 P55-C-FOS PROTO-ONCOGENE PROTEIN {Incyte PD:341491}
2.9 Human extracellular protein (S1-5) mRNA, complete cds {IncytePD:1798209}
2.8 Early growth response 2 (Krox-20 (Drosophila) homolog) {Incyte PD:3603037}
2.7 Human G0S3 mRNA, complete cds{Incyte PD:1998594}
2.6 serum-inducible kinase {Incyte PD: 1255087}
2.5 Human mitotic centromere-associated kinesin mRNA, complete cds {Incyte PD:2242674}
2.5 Homo sapiens ZW10 interactor Zwint mRNA, complete cds{Incyte PD: 1576329}
2.5 forkhead (Drosophila)-like 16{Incyte PD: 1516301}
2.4 KIAA0008 gene product {Incyte PD: 1970111}
2.3 thymidine kinase 1, soluble{Incyte PD: 2055926}
2.3 RAD54 (S.cerevisiae)-like{Incyte PD: 2645840}
2.3 serine/threonine kinase 12{Incyte PD: 161207}
2.2 phorbolin (similar to apolipoprotein B mRNA editing protein) {Incyte PD: 212573}
2.1 high-mobility group (nonhistone chromosomal) protein 2 {Incyte PD: 2916753}
2.1 ligase I, DNA, ATP-dependent{Incyte PD: 1841920}
2.1 KIAA0165 gene product{Incyte PD: 1668131}
2.1 keratin 6B {Incyte PD: 979835}
2.1 thymidylate synthetase {IncytePD: 39817}
2.1 T-cell receptor, gamma cluster{Incyte PD: 1857652}
2.1 specificity phosphatase 5{Incyte PD: 1734561}
2.1 Human mRNA for KIAA0074 gene, partial cds {Incyte PD: 4003342}
2.1 trophinin-assisting protein (tastin) {Incyte PD: 1340504}
2.1 ESTs, Highly similar to C8 [H.sapiens] {Incyte PD: 44938}
2.1 minichromosome maintenance deficient (S. cerevisiae) 2(mitotin) {Incyte PD: 1723834}
2.0 early growth response 3{Incyte PD: 2633001}
2.0 myeloid cell leukemia sequence1 (BCL2-related) {Incyte PD: 1842870}
2.0 histone acetyltransferase 1{Incyte PD: 3485789}
2.0 cell division cycle 2, G1 to S and G2 to M {Incyte PD: 1525795}
2.0 v-myb avian myeloblastosis viral oncogene homolog-like 2{Incyte PD: 494905}
2.0 ESTs, Highly similar to MITOTIC KINESIN-LIKE PROTEIN-1 [H.sapiens] {Incyte PD: 2640427}
1.9 inhibin, beta B (activin AB beta polypeptide) {Incyte PD: 1504993}
1.9 highly expressed in cancer, rich in leucine heptad repeats{Incyte PD: 1677406}
1.9 kinesin-like 2 {Incyte PD: 1562519}
1.9 serine/threonine kinase 15{Incyte PD: 2007691}

2. Experimental methods and procedures. Our previous progress report included in considerable detail descriptions of the experimental methods used in our studies. Procedures not previously described are presented here.

a. Isolation CTr from RXM. RXM (200 mg) was dissolved in 1 ml of THF and fractionated initially by silica gel vacuum liquid chromatography. Mixtures of hexane/THF with increasing polarity were used as mobile phase to obtain the following five crude fractions: 100% hexane (A), hexane/THF 2:1 (B), hexane/THF 1:1 (C), hexane/THF 1:2 (D) and 100% THF (E). Fractions B and C, shown by analytical HPLC to contain CTr, were combined, extracted with hexane to remove lipophilic components and resuspended in THF before injection onto semipreparative HPLC. HPLC purification of CTr was performed using a Shimadzu HPLC system (SCL-10A, Shimadzu Scientific Instruments, Inc., Japan) equipped with a C-18 bonded-phase semi-preparative column (Beckman Ultrasphere-ODS, 10 x 250 mm, 5 mm) (Beckman, San Ramon, CA) and UV/VIS detector (SPD-10AV, Shimadzu Scientific Instruments, Inc., Japan). Isocratic elution employed a mixture of acetonitrile/water (60:40) at a flow rate of 1.5 mL/min. and the detector setting was at 280 nm. The electron impact mass spectrometry analyses of the HPLC fractions of interest were performed at the Mass Spectrometry Facility of the College of Chemistry, University of California at Berkeley. The isolated CTr gave a single peak on analytical HPLC with a retention time of 44.9 min. and the expected mass spectrum.

b. Modeling of CTr binding to the ER ligand-binding domain. Since CTr is a strong agonist of estrogen receptor function but exhibits no obvious structural similarity to E_2, we compared the structure of CTr to other classes of ER ligands. Our comparisons included size or "steric" considerations and also electrostatic charge distribution observations. Quantum mechanical geometry optimizations were performed on 17β-estradiol, raloxifene, tamoxifen, and CTr molecules; solvent-accessible surfaces were then constructed surrounding these molecules to enable the solution of the Poisson-Boltzmann equation following the method of Wilson and associates (30). The electronic distribution of the molecule is allowed to rearrange in response to the polarization of its interface with an aqueous environment. The polarization charge induced on these surface elements is mapped onto the nodes ("dots") from which the surface is comprised. These calculations were performed at the 6-31G**/MP2 level of theory allowing for an accurate depiction of the induced surface charge density.

We next placed each of these molecules into the experimentally determined ER binding sites obtained by Brzozowski, et. al. and Shiau, et. al. from their complexing of the ER with the specific ligand and determining the respective crystal structure (31, 32). The minimum energy configurations of the molecules were determined by varying both the positions of their centers of mass and their three-dimensional angular orientation within the binding sites. Atoms comprising the binding site itself were not allowed to relax. Interatomic potentials required for this minimization were determined by pairwise summation using local density methods and reflect the electronic overlap repulsion between the atoms comprising the molecule and those in the appropriate receptor binding site.

Conclusions:

We have made several significant observations during this phase of the grant.

a) Our results show that at relatively high concentrations DIM can induce apoptosis in cultured breast tumor cells by a mechanism the is different from that of taxol, a promising beast cancer therapeutic agent.

b) We discovered that CTr, a major in vivo product of I3C, is a strong agonist of ER function and, thus, is a new class of phytoestrogens. When combined with our previous finding that DIM can also activate that ER pathway, this observation provides further evidence that oral I3C may function in part as an estrogen.

c) Ascorbigen and NI3C, two major naturally occurring derivatives of I3C are relatively weak activators of the Ah receptor pathway, in vivo, and thus are not likely to activate adverse Ah receptor-mediated adverse effects.

d) Very importantly, a series of genes involved in tumor angiogenesis were strongly down regulated in breast tumor cells treated with DIM. This observation provides a new rationale to explain the mode of action to this I3C product as a strong inhibitor of growth of established mammary tumors.

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FIGURE 1

National Cancer Institute Developmental Therapeutics Program Dose Response Curves	NSC: 708486 -A / 1	SSPL:	Exp. ID: 9905NS48-17
	Report Date: June 8, 1999	Test Date: May 25, 1999	

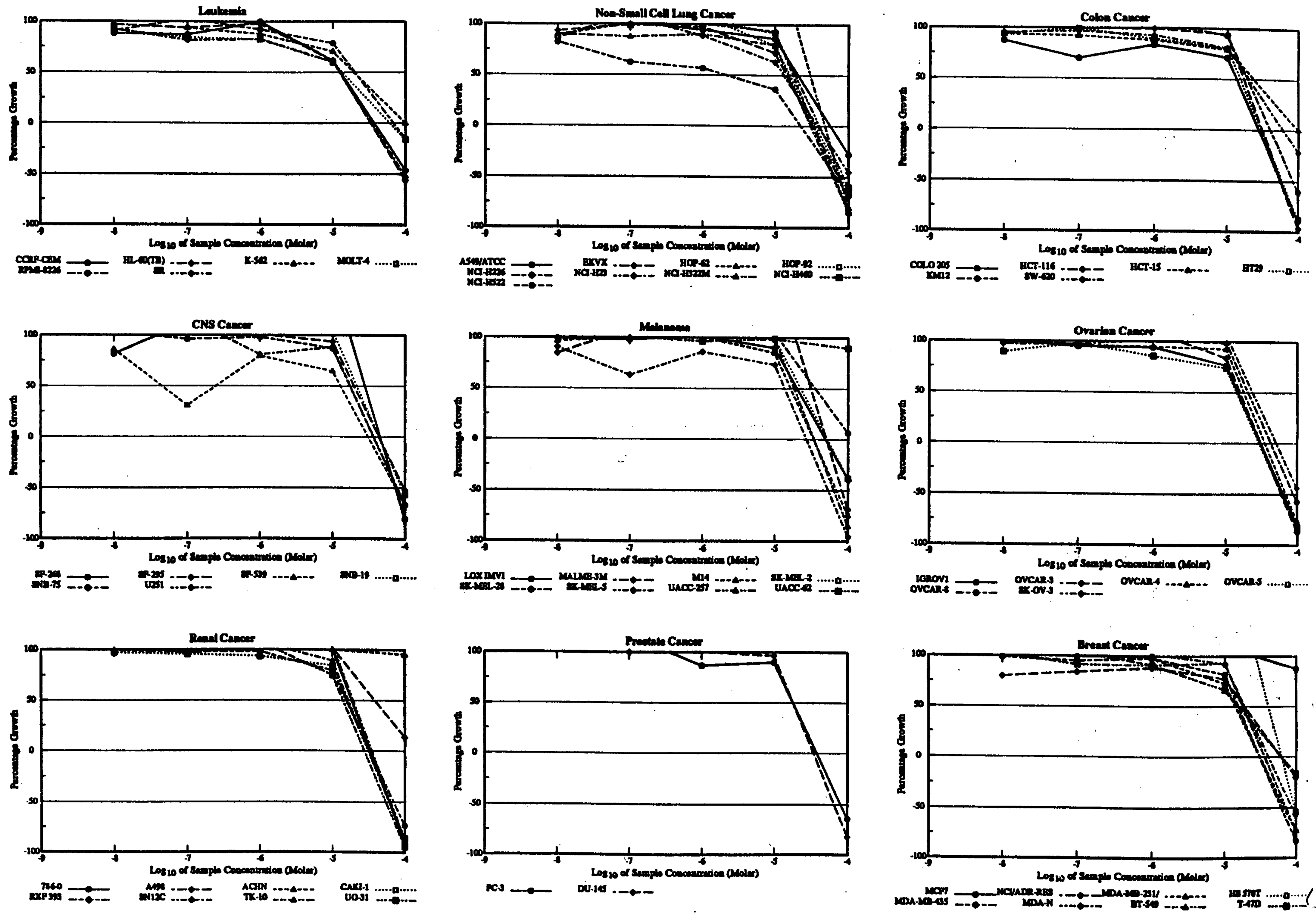


FIGURE 2

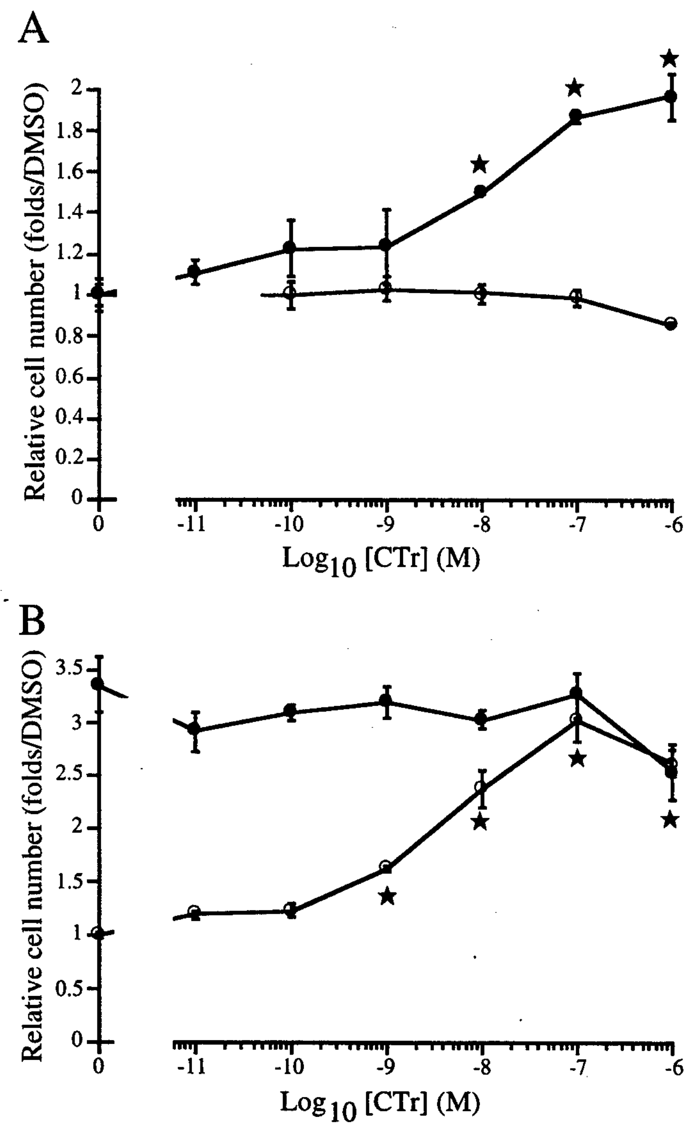
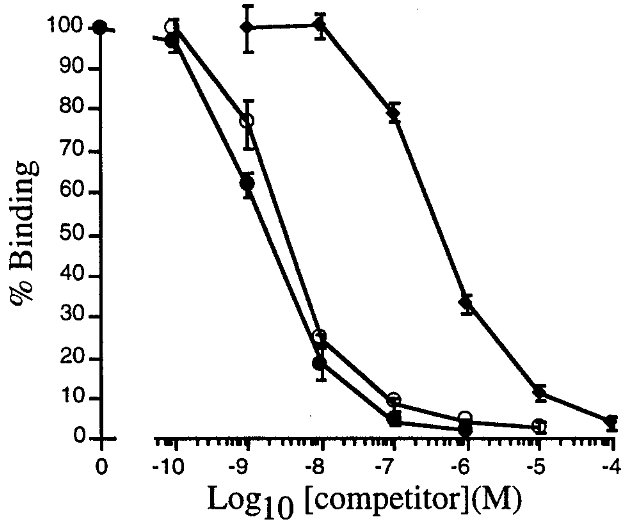

A
Relative cell number (folds/DMSO)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
-11
-10
-9
-8
-7
-6
Log10 [CTr] (M)
B
Relative cell number (folds/DMSO)
3.5
3
2.5
2
1.5
1
0.5
0
0
-11
-10
-9
-8
-7
-6
Log10 [CTr] (M)

FIGURE 3



100
90
80
70
60
50
40
30
20
10
0
% Binding
0
-10
-9
-8
-7
-6
-5
-4
Log10 [competitor](M)

FIGURE 4

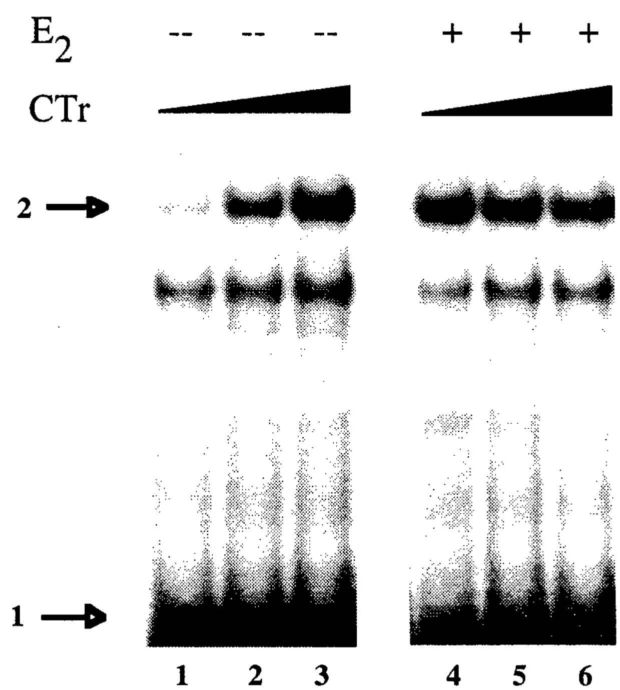


FIGURE 5

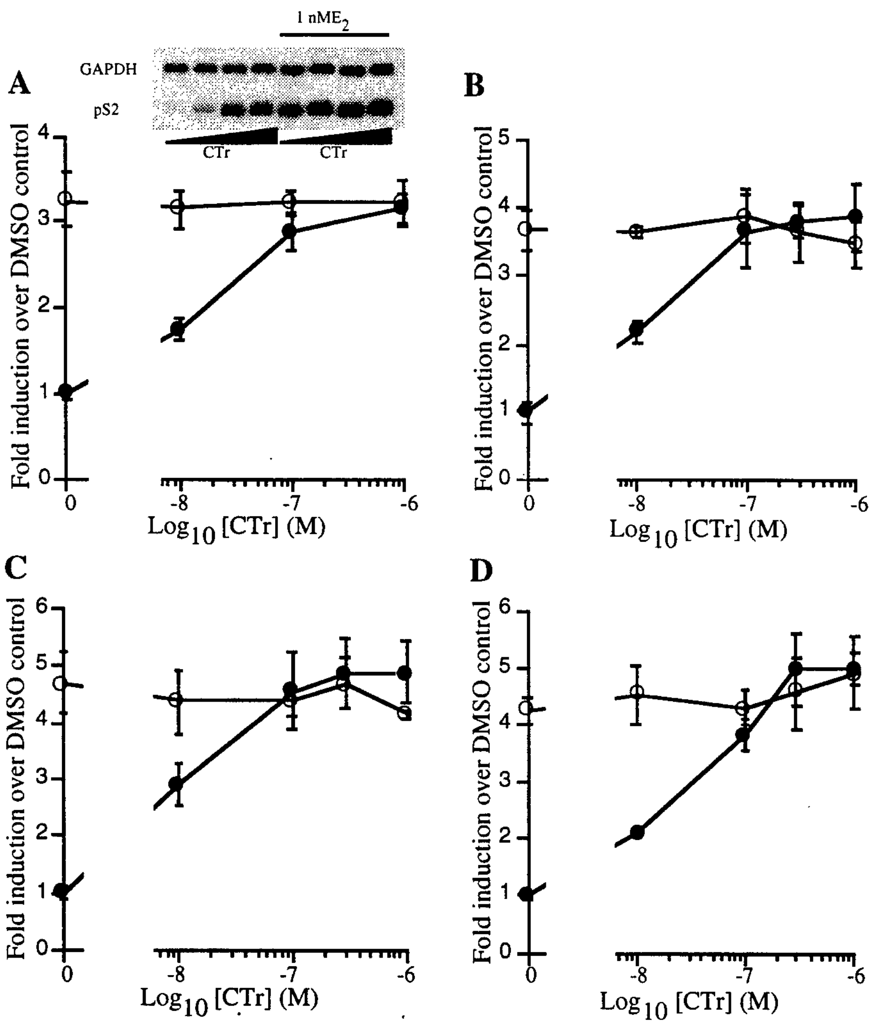

1 nME2
GAPDH
pS2
CTr
CTr
A
B
C
D
Fold induction over DMSO control
Log10 [CTr] (M)

FIGURE 6

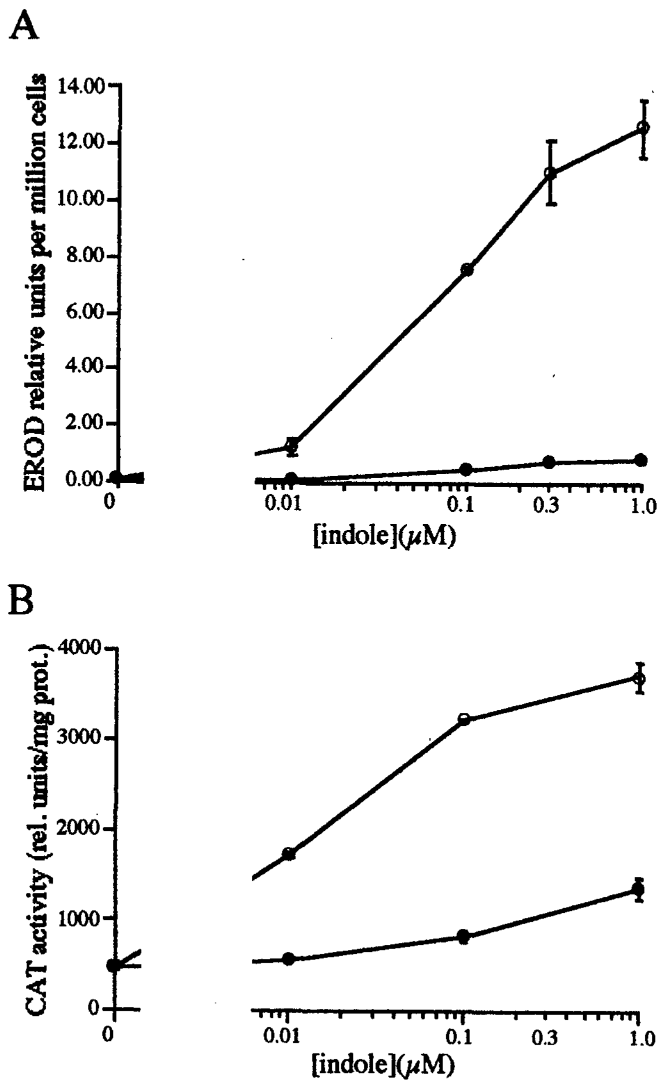

A
EROD relative units per million cells
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
0
0.01
0.1
0.3
1.0
[indole](μM)
B
CAT activity (rel. units/mg prot.)
4000
3000
2000
1000
0
0
0.01
0.1
0.3
1.0
[indole](μM)

FIGURE 7

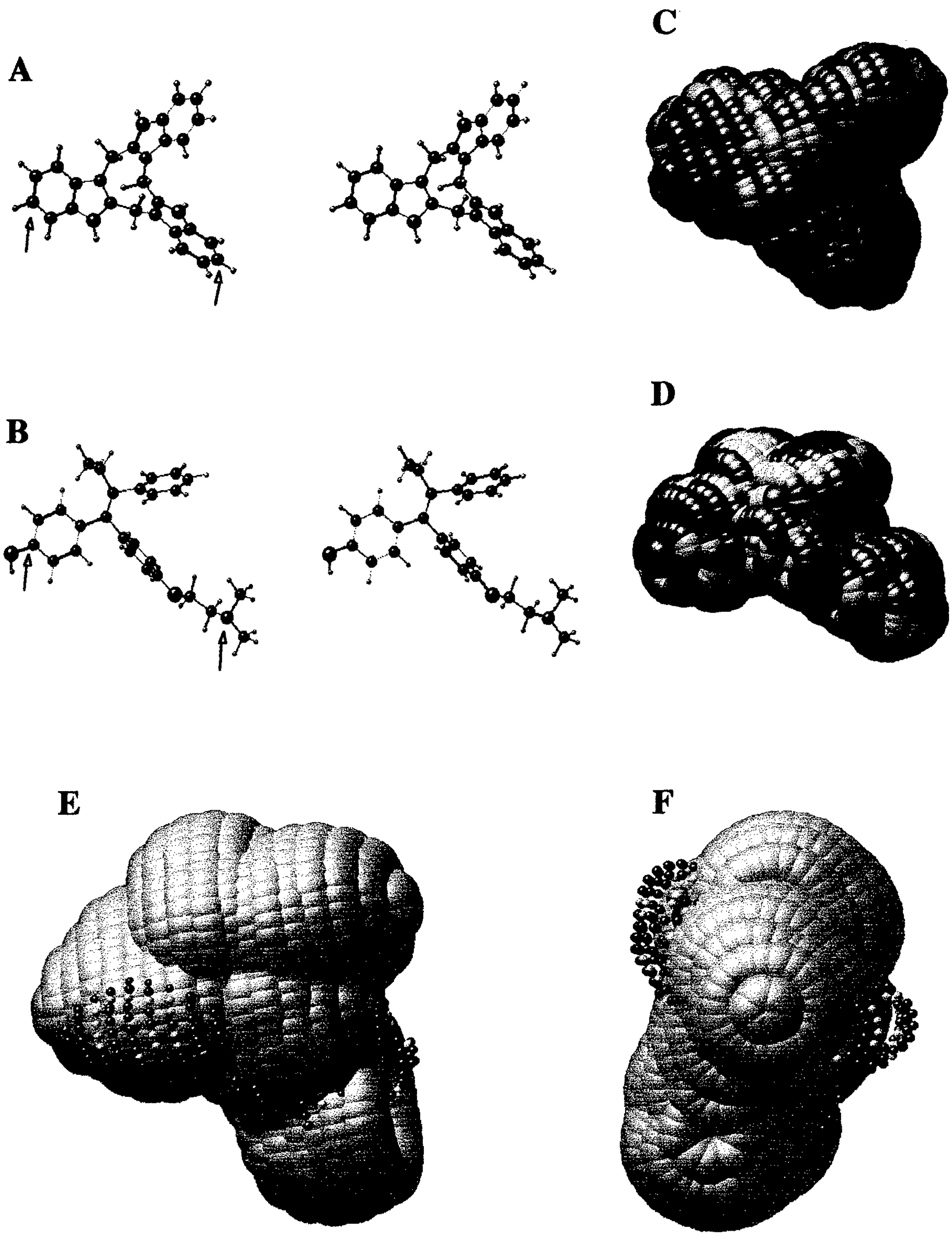
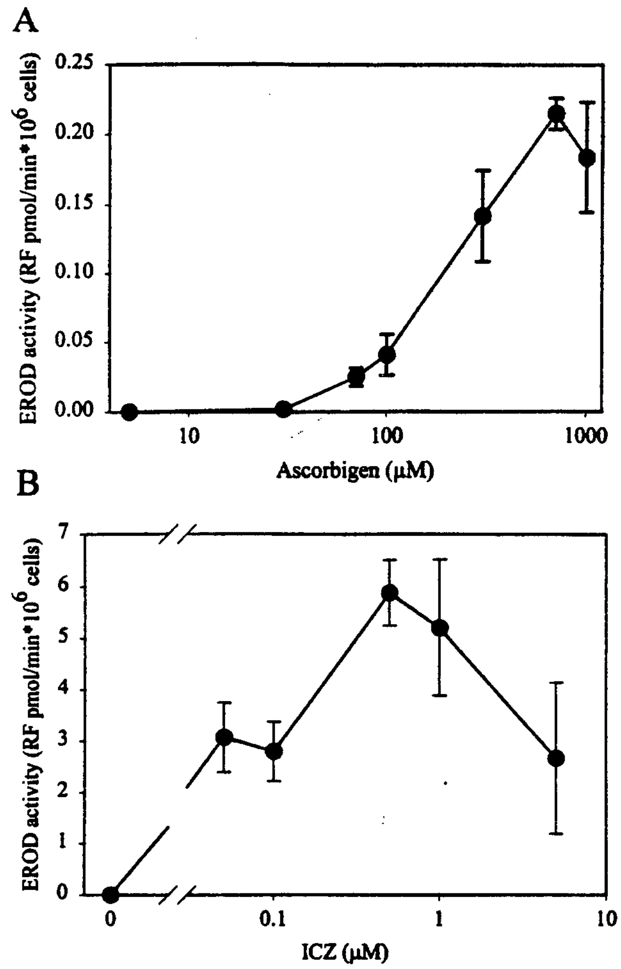
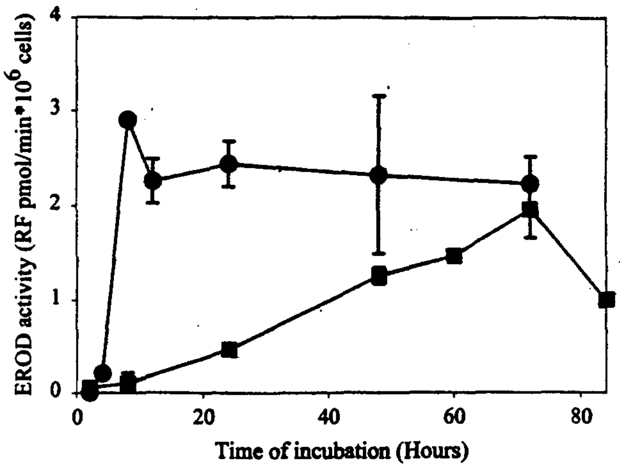


FIGURE 8



Effect of ASG and ICZ on EROD activity in Hepa 1c1c7 cells. Cells were treated with increasing concentrations of ASG (1–1000 μM), panel A, or ICZ (0.05–5 μM), panel B, for 24 hr. The cells were then harvested for analysis of enzyme activity. Activity induced by solvent (DMSO) was subtracted for each concentration point. A positive control (1 μM ICZ) was included in the ASG experiment (panel A), i.e. 3.0 RF pmol/min/10^6 cells. Bars indicate mean values of three (panel A) or two (panel B) measurements ± SD (panel A) or range (panel B). The ASG induction experiment (panel A) was conducted twice with similar results.

FIGURE 9



Kinetics of EROD induction by ASG dissolved in DMSO or PBS in Hepa 1c1c7 cells. The cells were treated with 700 µM ASG dissolved in either DMSO (■) or PBS (●) and harvested at designated time points for analysis of enzyme activity. Activity induced by solvent (DMSO or PBS) was subtracted for each concentration point. Bars indicate mean values of two measurements ± range. The experiments were conducted twice with similar results.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 23 Aug 01

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Phylis Rinehart

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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